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Citation for published version:

Kuhnle, G, Heinze, A, Schmitt, J, Giesow, K, Taylor, G, Morrison, I, Rijsewijk, FAM, Van Oirschot, JT & Keil, GM 1998, 'The class II membrane glycoprotein G of bovine respiratory syncytial virus, expressed from a synthetic open reading frame, is incorporated into virions of recombinant bovine herpesvirus 1', *J Virol*, vol. 72, no. 5, pp. 3804-11.

Link:

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Document Version:

Publisher's PDF, also known as Version of record

Published In:

J Virol

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J. Virol. 1998, 72(5):3804.

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The Class II Membrane Glycoprotein G of Bovine Respiratory Syncytial Virus, Expressed from a Synthetic Open Reading Frame, Is Incorporated into Virions of Recombinant Bovine Herpesvirus 1

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Received 22 August 1997/Accepted 16 January 1998

The bovine herpesvirus 1 (BHV-1) recombinants BHV-1/eG_{ori} and BHV-1/eG_{syn} were isolated after insertion of expression cassettes which contained either a genomic RNA-derived cDNA fragment (BHV-1/eG_{ori}) or a modified, chemically synthesized open reading frame (ORF) (BHV-1/eG_{syn}), which both encode the attachment glycoprotein G of bovine respiratory syncytial virus (BRSV), a class II membrane glycoprotein. Northern blot analyses and nuclear runoff transcription experiments indicated that transcripts encompassing the authentic BRSV G ORF were unstable in the nucleus of BHV-1/eG_{ori}-infected cells. In contrast, high levels of BRSV G RNA were detected in BHV-1/eG_{syn}-infected cells. Immunoblots showed that the BHV-1/eG_{syn}-expressed BRSV G glycoprotein contains N- and O-linked carbohydrates and that it is incorporated into the membrane of infected cells and into the envelope of BHV-1/eG_{syn} virions. The latter was also demonstrated by neutralization of BHV-1/eG_{syn} infectivity by monoclonal antibodies or polyclonal anti-BRSV G antisera and complement. Our results show that expression of the BRSV G glycoprotein by BHV-1 was dependent on the modification of the BRSV G ORF and indicate that incorporation of class II membrane glycoproteins into BHV-1 virions does not necessarily require BHV-1-specific signals. This raises the possibility of targeting heterologous polypeptides to the viral envelope, which might enable the construction of BHV-1 recombinants with new biological properties and the development of improved BHV-1-based live and inactivated vector vaccines.

Bovine herpesvirus 1 (BHV-1), a member of the subfamily *Alphaherpesvirinae* with a double-stranded DNA genome of approximately 136 kbp, causes infectious rhinotracheitis and infectious pustular vulvovaginitis as the most common clinical symptoms in cattle (27, 34, 39). Vaccination with attenuated live viruses or inactivated virions is widely used to control the disease and to reduce the concomitant financial losses. As with other large DNA viruses, interest exists in the use of recombinant BHV-1 as an improved live vaccine against BHV-1 infection (1, 21, 42) or as a vector for bi- or multivalent vaccines against BHV-1 and additional bovine pathogens (17, 18). To date, incorporation of heterologous genes into the genome of BHV-1 has concentrated mainly on the expression of the procaryotic *lacZ* gene to identify essential and nonessential genes or as a reporter gene for analytical studies (3, 8, 12, 15, 20, 29, 37, 38, 45). Recently, BHV-1 has been used to express biologically active bovine interleukins (21, 32) and glycoproteins of pseudorabiesvirus (19, 31). However, expression of RNA virus-encoded proteins by BHV-1 has not been published so far. Remarkably, expression of genes from cytoplasm-replicating viruses by other herpesviruses of mammals has only rarely been reported (5, 43).

Attempts to express the fusion glycoprotein F and the at-

tachment glycoprotein G of bovine respiratory syncytial virus (BRSV), a pneumovirus of the family *Paramyxoviridae* which is also prevalent worldwide and causes severe respiratory disease in young calves similar to the disease caused by human respiratory syncytial virus in children (4), were not successful (13, 33). Although the cDNA fragments encoding the respective glycoproteins were flanked by transcription control elements that are active in the genomic context of BHV-1 (21), no BRSV-specific transcripts were detected in cells infected with the BHV-1 recombinants (13) (see below). We therefore assumed that RNAs containing the authentic BRSV sequences were unstable in the nuclei of infected cells. To test this assumption, the BHV-1 glycoprotein D (gD) codon usage preferences (13, 40) were used to construct a modified open reading frame (ORF) encoding the BRSV G glycoprotein by chemically synthesized oligonucleotides.

In this report, we show that expression of the attachment glycoprotein G of BRSV (BRSV G glycoprotein), a type II membrane glycoprotein (36, 44), by BHV-1 was dependent on the modification of the base composition of the ORF encoding BRSV G glycoprotein, that virions produced by the recombinant contained the BRSV G glycoprotein, and that the presence of this protein in the viral envelope does not significantly interfere with the infectivity of BHV-1. Our findings suggest that RNA viruses which replicate in the cytoplasm can contain sequences or sequence elements that lead to instability of transcripts within the nucleus.

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MATERIALS AND METHODS

Cell culture and viruses. BHV-1 strain Schönböken (BHV-1/Schö) was obtained from O. C. Straub (Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany) and propagated on Madin-Darby bovine kidney cell clone Bu100 (MDBK-Bu100; kindly provided by W. Lawrence and L. Bello, University of Pennsylvania, Philadelphia, Pa.). The cells were grown in Dulbecco's minimum essential medium supplemented with 5% fetal calf serum (FCS), 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 0.35 mg of L-glutamine per ml. The gD-negative mutant BHV-1/80-221 was propagated on the constitutively gD-expressing cell line BU-Dorf as described previously (38).

Construction and cloning of the BRSV G_{syn} ORF. Oligonucleotides were synthesized in a Bioscience 8700 instrument and purified by gel electrophoresis. Complementary oligonucleotides were mixed in equal molar amounts in 10 mM Tris-HCl (pH 7.5), boiled for 5 min, and slowly cooled to room temperature. All the cloning procedures were performed by established methods (35). The following double-stranded DNA fragments were generated (restriction enzyme cleavage sites used for cloning are shown in boldface type).

Fragment 1:

AAGCTTACAAGTATGAGCAACACACGACACGACACCACTGAAGTTCAAGACGCTGAAGTGCAT**TTTCAAT**GTTCATACGTTGGTGTGCGTGTGCGTGTGACTCAAGTTCTGCGACTTC
HindIII

CGCGCTGGAAGGCTAGCAAGTACTTTCATCGTCGCGCTGAGCTGCCTGTACAAGTTCAACCTGA
GCGCGCACCTTCGATCGTTTATGAAGTAGCAGCGGACTCGACGGACATGTTCAAGTTGGACT

AGACCTGCTCCAGACGGCGCTGAGCAGCT**TCGCGAG**
TTCGACACAGGCTGTCCGCGACTCGTGC**AGCGCT**CTCTAG
NruI

Fragment 2:

CGATGATCAGCTGACGAGCGCTGTGTCATCAGCGGATCATCTACATCTCCGTGGGCAACGCG
GCTACTAGTCGCGACTGCTCGGACAGTAGTGCCGCTAGTAGATGATAGGACACCGTGTGCGC

AAGCGAAGCG**CGAGCTCG**
TTCGCTTCCGCT**CGAGCTCG**CTAG
AatII

Fragment 3:

CGAAGCCGACGATCCAGCAGACGACGAGCGCGAAGACACAGAGCCGCTTCTTCAAGG
TGCATCTCGGCTGCTAGGTGCTGCTGCTGCGCGCTTGGTGTGCTCGGCGAAGAGTGCC

AGCACAACCTACAAGAGCAGCAGCAGCATCCAGAGCAGCACT**TAAG**
TCGTGTGATGTTCTGTCGCTGTGCTGCTAGGTCTCGTGTGCT**GAATTC**CTAG
AflII

Fragment 4:

GCTTAAGCAGCTGCTGAACATCGACACGACGCGCGGCTATCGTATGGCCACAGCAGCA
ACGTC**GAATTC**GGTTCAGCACTGTAGCTGTGCTGCGCGCGTAGTCATACCGGTGTGCTGCT

ACGAGCCGAGAACCGAAGATCAAGCGCAGAGCAGCTGCGCGCGACGCGCAAGCCGCGGAT
TGCTCTGCGTCTTGGCGTTCTAGTTTCCGGTCTGTCGACGGCGCTGCGCGTTCGGCGGCTA
CAATCCGAGCGGAT
GTTGGCTCGCTAGC

Fragment 5:

GAAGCT**TATCGAT**ACCGCGGAGAACACAGGACCAACAACCTTCCAGACGCTGCC
ACGCTTCCGA**ATAGCT**ATGGCGGCTCTTGGTGTGCTGCTGTTGTTGAAGTCTGCGACGG
ClaI

GTACGTCCTCGTGCAGCAGCTGCGAGG
CATGACGGGACGCTGTCAGCTCCACTAG

Fragment 6:

GGTAACCTGGCGTGCCTGAGCTGTGCCACATCGAGACGGAGCGCGCGGAGCCGGG
ACGTC**CATTGGA**ACCGCAGCACTCGACACGCTGAGCTTGCCTGCGCGCGCTGCGGCC
BstEII

CCCCGACGATCAGCTGAAGAAGACCGCGAAGCGAAGACGACGAAGAAGCCGACGAAGACG
GGGGCTGCTAGTGACATCTTCTGCGGCTTCTGCTGCTTCTCGGCTGCTCTGCTG

ACGATCCACCGCA
TCTAGTGGTGGCGTGATC

Fragment 7:

GACTAGTCCGAGACGAAGCTCCAGCCGAAGAACACGCGCAGCGCAGCAAGGC
ACGCT**TGATCAG**GGCTCTGCTTCGAGGTGCGCTTCTGTTGTGCGCTGCGCGCTGCTTCCG
SpeI

ATCTGAGCAGCAGGAGCACCACGAACGAGCAGCAGCAGATCT**GAATTC**CA
TAGGACTCGTGCCTCTGCTGTGCTTGGTCTGCTGCTGCTGCTAGACT**TAAGTT**CGA
EcoRI

Fragment 1 was ligated into plasmid vector pUC18 after cleavage with *AatII* and *BamHI*. In the resulting plasmid, pF1, the *AatII* cleavage site of pUC18 was destroyed. Plasmid pF1 was cleaved with *NruI* and *BamHI* and received fragment 2 to produce pF2, into which fragment 3 was ligated after digestion with *AatII* and *BamHI* to yield pF1-3. In parallel, pUC19 DNA was cleaved with *HindIII* and *PstI*, fragment 7 was inserted, and the resulting plasmid, pF7, was cleaved with *PstI* and *SpeI* and received fragment 6 to generate pF6. Plasmid pF6 was incubated with *PstI* and *BstEII*, and fragment 5 was inserted. The plasmid obtained, pF5, was treated with *PstI* and *ClaI*, and fragment 4 was integrated, resulting in plasmid pF4. Plasmid pF4 was cleaved with *XbaI*, blunt ended with Klenow polymerase, and resealed with *AflII*. Into this DNA, the *HindIII*-*AflII* fragment of pF1-3 was ligated after blunt ending of the *HindIII* site. The resulting plasmid, pBRSVG_{syn}, contained the reconstructed BRSV G ORF flanked by *EcoRI* cleavage sites.

Other plasmid constructions. Plasmid pRSV02 (kindly provided by Paul Sondermeijer, Intervet International, Boxmeer, The Netherlands), which contains a

cDNA fragment encoding the BRSV G glycoprotein, was cleaved with *BglII*. A 770-bp fragment, which encompasses codons 1 to 257 of the BRSV G ORF, was ligated into plasmids pROMie and pROMe (21) cleaved with *BglII* followed by insertion of a synthetic DNA fragment, obtained by hybridizing oligonucleotides 5'-CATGCACACCTCCATATAA-3' and 5'-CATGTTATATGGAGGTGTG-3', as described above, into a single *NcoI* cleavage site to provide a stop codon. The resulting plasmids, in which codon 259 was changed from CAA (encoding glutamine) to ATG (encoding methionine), were named pROMieG_{ori} and pROMeG_{ori}, respectively.

To construct recombination plasmid pROMeG_{syn}, plasmid pROMe was cleaved with *BglII*, blunt ended with Klenow polymerase, and used for the insertion of the synthetic G ORF, isolated from plasmid pBRSVG_{syn} after cleavage with *EcoRI* and blunt ending with Klenow polymerase. For synthesis of cRNA, the same fragment was integrated into the *BglII*-cleaved and blunt-ended plasmid vector pSP73 (Promega, Heidelberg, Germany) to give plasmid pSPG_{syn}. For in vitro transcription of the BRSV G_{ori} ORF, a blunt-ended *ClaI* fragment encompassing this ORF in plasmid pROMeG_{ori} was inserted into *EcoRV*-cleaved pSP73. The resulting plasmid was designated pSPG_{ori}. The correctness and orientation of the BRSV G ORFs were determined by dideoxy sequencing, which showed that the 5' ends of the G_{ori} and G_{syn} ORFs were placed adjacent to the T7 or SP6 promoter, respectively.

In vitro transcription and translation. Plasmids pSPG_{ori} and pSPG_{syn} were linearized with *BglII* and *HindIII*, respectively, and cRNA was transcribed by T7 or SP6 RNA polymerase in the presence of the cap analog m⁷GpppG as specified by the manufacturer (Boehringer GmbH, Mannheim, Germany). In vitro translation of the cRNAs was performed in the presence of 60 µCi of [³⁵S]methionine per reaction mixture as recommended by the supplier (Promega). Labeled proteins were visualized by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) as described previously (16).

RNA isolation, Northern blot hybridization, and nuclear runoff analysis. Whole-cell RNA and cytoplasmic RNA was isolated as described previously (14, 35). Glyoxal-treated RNA (5 µg) was separated in 1% formaldehyde gels, transferred to nitrocellulose filters, and hybridized to ³²P-labeled DNA by established procedures (14, 35). Nuclear runoff transcription assays were performed by the method of Greenberg and Ziff (11). Labeled RNA was hybridized to equimolar (1.5 pmol) amounts of plasmid DNA dotted onto nitrocellulose filters with a dot blot device (Schleicher & Schüll, Dassel, Germany). Bound radioactivity was visualized by autoradiography and quantitated by Cerenkov counting.

Antibodies and sera. BRSV G glycoprotein-specific monoclonal antibody (MAb) 20 and MAb 57 and the polyclonal serum directed against BHV-1 gD are described elsewhere (8, 9, 23). The anti-VacG_{syn} serum was raised in rabbits infected with a recombinant vaccinia virus expressing the G_{syn} ORF under control of the p7.5 promoter. The rabbits were inoculated with 5 × 10⁷ PFU and bled 4 weeks after a booster immunization with 5 × 10⁷ PFU given 1 week after the initial infection. The BRSV-neutralizing titer was 1:80 in the presence of complement. Polyclonal anti-BRSV hyperimmune serum 2106 was raised in gnotobiotic calves after repeated inoculation with BRSV and had a BRSV-neutralizing titer of 1:10,000 in the absence of complement (10).

Western blotting and immunoprecipitation. For Western blotting (immunoblotting), cells or purified virions were lysed in sample buffer. For analysis of secreted proteins, infected cells were cultured with Dulbecco's minimal essential medium lacking FCS. Culture supernatants were clarified by ultracentrifugation and, after addition of 3 volumes of ice-cold acetone, incubated for 15 min at -70°C. Precipitates were collected by centrifugation, air dried at room temperature, and resuspended in sample buffer. The proteins were separated and immunodetected as described previously (37). [³⁵S]methionine-labeled proteins were immunoprecipitated from purified virions as described by Fehler et al. (8). The apparent molecular masses of proteins in SDS-polyacrylamide gels were determined from standard curves with ¹⁴C-methylated protein molecular mass standards (Amersham, Braunschweig, Germany) or prestained protein molecular mass standards (Gibco BRL, Eggenstein, Germany).

Deglycosylation reactions. Virions, purified as described previously (8), were resuspended in 0.5% Nonidet P-40 in phosphate-buffered saline (PBS) and incubated overnight at 37°C with 0.4 U of N-glycosidase F (Boehringer), 1 mU of neuraminidase (Boehringer) or 1 mU of neuraminidase and 1.5 mU of O-glycosidase (Boehringer). Immunoprecipitated proteins were incubated with the respective enzymes under conditions recommended by the supplier.

Virus neutralization assays. Samples (approximately 200 PFU) of wild-type and recombinant BHV-1 strains were incubated with serial dilutions of MAbs or sera in a final volume of 100 µl of cell culture medium containing 20% FCS with or without 5% normal rabbit serum as a source of complement and plated onto MDBK-Bu100 cells after 1 h at 37°C. The cultures were overlaid with semisolid medium 2 h later, and plaques were counted 2 to 3 days after infection. The percent neutralization-resistant infectivity was calculated from the results with controls incubated without antibody.

Infection of cells for indirect immunofluorescence. MDBK-Bu100 cell cultures were infected with approximately 100 PFU and overlaid with semisolid medium. After the development of plaques, the cultures were fixed with 3% paraformaldehyde in PBS and sequentially incubated with appropriate dilutions of MAbs or sera and 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF)-conjugated goat anti-species immunoglobulin G (Dianova, Hamburg, Germany).

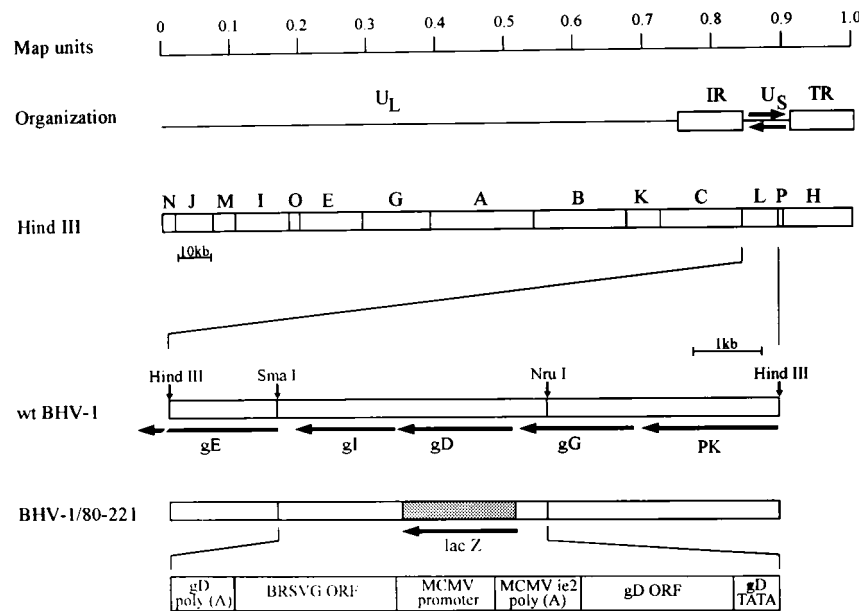


FIG. 1. Construction of BHV-1 recombinants. The schematic representation of the prototype orientation of the BHV-1 genome is shown above the *Hind*III restriction fragment map of BHV-1 strain Schönböken (7, 27). The wild-type *Hind*III L fragment is enlarged, and the location and direction of transcription of genes encoding the putative protein kinase (PK) and glycoproteins G (gG), D (gD), I (gI), and E (gE) are indicated by arrows (15, 25, 40, 45). Relevant restriction enzyme cleavage sites are marked. The respective *Hind*III fragment of the *gD*⁻ *lacZ*⁺ mutant BHV-1/80-221 is depicted below. The location and direction of transcription of the *lacZ* cassette (dotted area, not drawn to scale) that replaces the gD ORF is indicated by an arrow. Underneath, a diagram of the integration fragment contained in the recombination vectors is shown. The gD TATA and gD poly(A) segments indicate BHV-1 sequences representing the gD promoter and the gD polyadenylation signal, respectively, which provide homologous regions for recombination. In this study, transcription of the BRSV *G*_{ori} or *G*_{syn} ORF (BRSVG ORF) was directed by the MCMV *ie1* enhancer-promoter element (6) in transient-expression experiments or by the MCMV *e1* promoter (2) in recombinant BHV-1 infected cells. Transcription of the BHV-1 gD ORF which is followed by the MCMV *ie2* poly(A) signal (28) is under the control of the authentic gD promoter.

RESULTS

Rationale for synthesis of a modified BRSV G glycoprotein ORF. To express the BRSV G glycoprotein by recombinant BHV-1, recombination plasmid pROME_{G_{ori}}, which contains a cDNA fragment encompassing the BRSV G glycoprotein ORF under control of the murine cytomegalovirus (MCMV) early 1 promoter (Fig. 1), was cotransfected with purified BHV-1/80-221 DNA into MDBK cells and the progeny virus was serially diluted and plated on MDBK cells. Since the ORF encoding the essential gD is replaced by a *lacZ* expression cassette in BHV-1/80-221 (8), only viruses which have acquired the gD ORF by replacement of the *lacZ* cassette by the insert of the recombination plasmid should be able to replicate on non-complementing cells (21). Virus from plaques that did not stain blue under a Bluo-Gal-containing agarose overlay were again subjected to titer determination on MDBK cells, and isolates which produced only "white" plaques were plaque purified once more and characterized further (see Fig. 4). Analysis of MDBK cells infected with the resulting recombinant BHV-1/eG_{ori} gave no indication of BRSV G glycoprotein expression by indirect immunofluorescence (see Fig. 7), and no stable transcripts were detected within cytoplasmic RNA (data not shown) or whole-cell RNA (see Fig. 5). BRSV G glycoprotein expression was also not detected in transient-expression experiments with plasmid pROMieG_{ori} (Fig. 1) in which the BRSV G glycoprotein ORF is under the control of the MCMV immediate-early 1 enhancer/promoter element (data not shown). In vitro translation of mRNA, transcribed in vitro from pSPG_{ori}, resulted in synthesis of a polypeptide whose apparent molecular mass of 31 kDa (Fig. 2, lane 2) was in good agreement with the calculated size of 29 kDa (24). This result indicated that mRNA from the BRSV *G*_{ori} ORF contained

within pROME_{G_{ori}} has the potential to direct protein synthesis, and we assumed that the failure to detect BRSV G glycoprotein-specific gene products was due to instability of BRSV G glycoprotein transcripts in the nuclei of BHV-1/eG_{ori}-infected cells. Therefore, a modified ORF encoding the BRSV G glycoprotein (Fig. 3) was chemically synthesized, isolated from plasmid pBRSVG_{syn}, and inserted into pROME and pSP73. The resulting plasmids were named pROME_{G_{syn}} and pSPG_{syn}, respectively, and RNA transcribed from pSPG_{syn} directed the synthesis of a 31-kDa protein in a rabbit reticulocyte lysate (Fig. 2, lane 3) that comigrated with the polypeptide synthesized in vitro from the *G*_{ori} ORF, supporting the conclusion

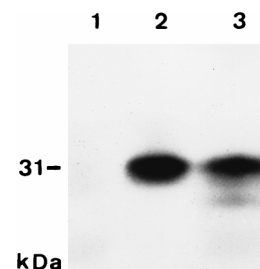


FIG. 2. Cell-free translation of cRNAs encompassing the BRSV *G*_{ori} or *G*_{syn} ORF. ³⁵S-labeled proteins translated in vitro from RNA transcribed in vitro from plasmids pSPG_{ori} (lane 2) or pSPG_{syn} (lane 3) were size separated in SDS-10% polyacrylamide gels and visualized by fluorography. In lane 1, proteins synthesized from RNA transcribed from pSPG_{ori} antisense to the BRSV G glycoprotein ORF were separated. The apparent molecular mass of the translation products, indicated in kilodaltons on the left, was calculated from the migration of ¹⁴C-methylated protein molecular mass standards run on the same gel.

1	ATG	TCC	AAC	CAT	ACC	CAT	CAT	CTT	AAA	TTC	AAG	ACA	TTA	AAG	AGG	GCT	TGG	AAA	GCC	TCA
1	M	S	N	H	T	H	H	L	K	F	K	T	L	K	R	A	W	K	A	S
61	AAA	TAC	TTT	ATA	GTA	GGA	TTA	TCA	TGT	TTA	TAT	AAG	TTC	AAT	TTA	AAA	TCC	CTT	GTC	CAA
21	K	Y	F	I	V	G	L	S	C	L	Y	K	F	N	L	K	S	L	V	Q
121	ACG	GCT	TTG	TCC	ACC	CTA	GCA	ATG	ATA	ACC	TTG	ACA	TCA	CTC	GTC	ATC	ACA	GCC	ATT	ATT
41	T	A	L	S	T	L	A	M	I	T	L	T	S	L	V	I	T	A	I	I
181	TAC	ATT	AGT	GTG	GGA	AAT	GCT	AAA	GCC	AAG	CCC	ACA	TCC	AAA	CCA	ACC	ATC	CAA	CAA	ACA
61	Y	I	S	V	G	N	A	K	A	K	P	T	S	K	P	T	I	Q	Q	T
241	CAA	CAG	CCC	CAA	AAC	CAT	ACC	TCA	CCA	TTT	TTC	ACA	GAG	CAC	AAC	TAC	AAA	TCA	ACT	CAC
81	Q	Q	P	Q	N	H	T	S	P	F	F	T	E	H	N	Y	K	S	T	H
301	ACA	TCA	ATT	CAA	AGC	ACC	ACA	CTG	TCC	CAA	CTA	CTA	AAC	ATA	GAC	ACT	ACT	AGA	GGA	ATT
101	T	S	I	Q	S	T	T	L	S	Q	L	L	N	I	D	T	T	R	G	I
361	ACA	TAT	GGT	CAC	TCA	ACC	AAC	GAA	ACC	CAA	AAC	AGA	AAA	ATC	AAA	GGC	CAA	TCC	ACT	CTA
121	T	Y	G	H	S	T	N	E	T	Q	N	R	K	I	K	G	Q	S	T	L
421	CCC	GCC	ACC	AGA	AAA	CCA	CCA	ATC	AAT	CCA	TCG	GGA	AGC	ATC	CCC	CCT	GAA	AAC	CAT	CAA
141	P	A	T	R	K	P	P	I	N	P	S	G	S	I	P	P	E	N	H	Q
481	GAC	CAC	AAC	AAC	TTC	CAA	ACA	CTC	CCC	TAT	GTG	CCT	TGC	AGT	ACA	TGT	GAA	GGT	AAT	CTT
161	D	H	N	N	F	Q	T	L	P	Y	V	P	C	S	T	C	E	G	N	L
541	GCT	TGC	TTA	TCA	CTC	TGC	CAT	ATT	GAG	ACG	GAG	AGA	GCA	CCA	AGC	AGA	GCC	CCT	ACA	ATC
181	A	C	L	S	L	C	H	I	E	T	E	R	A	P	S	R	A	P	T	I
601	ACC	CTC	AAA	AAG	ACT	CCA	AAA	CCC	AAA	ACC	ACT	AAA	AAG	CCA	ACC	AAG	ACA	ATC	CAC	CAC
201	T	L	K	K	T	P	K	P	K	T	T	K	K	P	T	K	T	T	I	H
661	CAC	AGA	ACC	AGC	CCT	GAA	ACC	AAA	CTG	CAA	CCT	AAA	AAC	AAC	ACA	GCA	ACT	CCA	CAA	CAA
221	H	R	T	S	P	E	T	K	L	Q	P	K	N	N	T	A	T	P	Q	Q
721	GGC	ATC	CTC	TCT	TCA	ACA	GAA	CAT	CAC	ACA	AAT	CAA	TCA	ACT	ACA	CAG	ATC	TAG		
241	G	I	L	S	S	T	E	H	H	T	N	Q	S	T	T	Q	I	*		

FIG. 3. Comparison of the nucleotide sequences of the BRSV G glycoprotein cDNA ORF (upper sequence) and the G_{syn} ORF (lower sequence). Exchanged nucleotides are in boldface type, and the deduced BRSV G glycoprotein amino acid sequence is shown in the single-letter code.

that the 31-kDa protein represents the primary translation product encoded by the BRSV G glycoprotein ORF.

Integration of the BRSV- G_{syn} ORF into the genome of BHV-1/80-221. Recombination plasmid pRomeG_{syn} was cotransfected with purified BHV-1/80-221 DNA into MDBK cells, and the recombinant virus, BHV-1/eG_{syn}, was isolated as described above for BHV-1/eG_{ori}. To demonstrate integration of the expression cassettes into the genomes of the respective viruses, MDBK cells were infected with BHV-1/eG_{ori}, BHV-1/eG_{syn}, and BHV-1/80-221, phenotypically complemented by propagation on gD-expressing cells (8). Whole-cell DNA was prepared 20 h postinfection (p.i.), cleaved with *Hind*III, transferred to nitrocellulose filters after size separation in 0.6% agarose gels, and hybridized to G_{syn} -, G_{ori} -, and *lacZ*-specific 32 P-labeled DNA fragments. The sizes of the fragments that hybridized to the respective probes were as expected (data not shown). We concluded that BHV-1/eG_{ori} and BHV-1/eG_{syn} were generated by homologous recombination as envisaged.

Transcription of the BRSV G_{ori} and BRSV G_{syn} ORFs in recombinant BHV-1-infected cells. To test for transcription from the recombinant BRSV G genes, MDBK cells were infected with BHV-1/eG_{syn} (Fig. 4, lanes 1, 3, 5, and 7) and BHV-1/eG_{ori} (lanes 2, 4, 6, and 8). Cytoplasmic RNA was isolated at 6 h p.i., size separated by agarose gel electrophoresis, and transferred to nitrocellulose filters. A 32 P-labeled DNA probe representing the BRSV G_{syn} ORF detected an RNA of 1.3 kb after infection with BHV-1/eG_{syn} (lane 1) and, after extended exposure, a transcript of 1.8 kb (lane 5) whose synthesis is initiated approximately 500 bp upstream from the

MCMV e1 promoter (13). Even after a longer exposure, G_{ori} ORF-specific transcripts of the expected size were not detected (lanes 2 and 6). The faint smear in lane 6 might indicate the presence of fragmented G_{ori} RNA. Hybridization of the filters with 32 P-labeled DNA encompassing the BHV-1 gD ORF showed that comparable amounts of gD mRNA were detected in BHV-1/eG_{syn}-infected cells (lanes 3 and 7) and BHV-1/eG_{ori}-infected cells (lanes 4 and 8), demonstrating that the

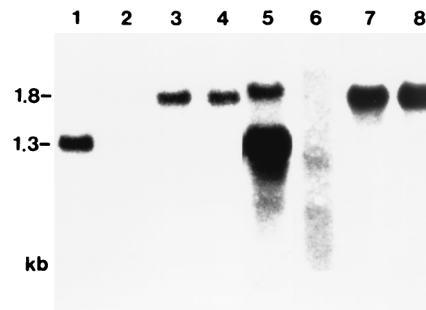


FIG. 4. Identification of transcripts encompassing the G_{syn} ORF. Whole-cell RNA from cells infected with BHV-1/eG_{syn} (lanes 1, 3, 5, and 7) and BHV-1/eG_{ori} (lanes 2, 4, 6, and 8) was prepared at 6 h p.i., and 5- μ g samples were transferred to nitrocellulose after 1% agarose gel electrophoresis. The filters were hybridized to 32 P-labeled DNA from the BRSV- G_{syn} ORF (lanes 1 and 5), the BRSV- G_{ori} ORF (lanes 2 and 6), and the BHV-1 gD ORF (lanes 3, 4, 7, and 8). Bound radioactivity was visualized by autoradiography. Lanes 5 to 8 are longer exposures of lanes 1 to 4. Transcript sizes are indicated in kilobases.

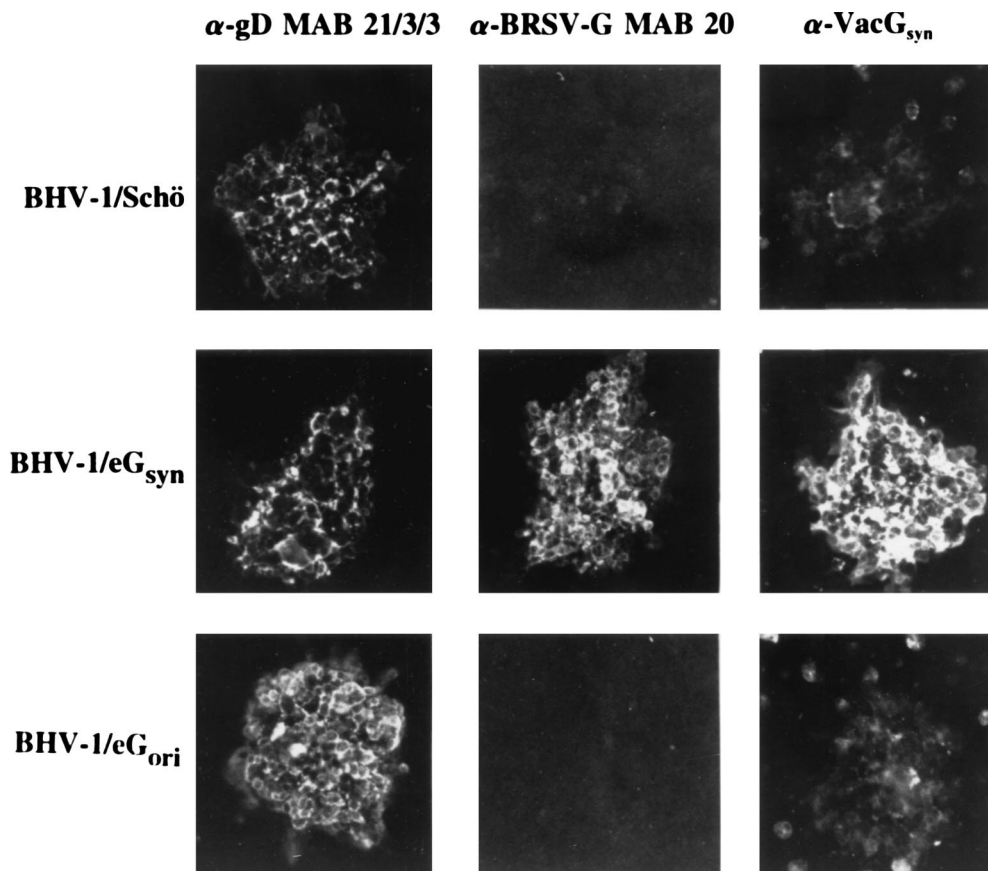


FIG. 5. BHV-1-encoded BRSV G glycoprotein is expressed on the cell surface. MDBK cell cultures were infected with approximately 100 PFU of BHV-1/Schö, BHV-1/eG_{syn}, and BHV-1/eG_{ori}, fixed after the development of plaques, and used for indirect immunofluorescence with BHV-1 gD-specific MAb 21/3/3, BRSV G glycoprotein-specific MAb 20, and the anti-VacG_{syn} serum. Bound antibodies were visualized by staining with DTAF-conjugated goat anti-species immunoglobulin G.

absence of stable transcripts from the BRSV G_{ori} ORF was not due to degradation of the RNA from BHV-1/eG_{ori}-infected cells. Substantially the same results were obtained with RNAs isolated 16 h after infection with BHV-1/eG_{syn} and BHV-1/eG_{ori} in the absence or presence of phosphonoacetic acid (data not shown).

To support the assumption that transcripts from the BRSV G_{ori} ORF are unstable in BHV-1/eG_{ori}-infected cells, elongation of initiated transcripts in BHV-1/eG_{ori}- and BHV-1/eG_{syn}-infected MDBK cells was analyzed by nuclear runoff transcription assays. ³²P-labeled RNA, synthesized in nuclei isolated at 6 h p.i., was hybridized to DNA from the plasmid vector pSP73 or from plasmids pSPG_{ori}, pSPG_{syn}, or pSPG_d dotted onto nitrocellulose membranes. Elongation of RNA from both BRSV G ORFs was detected (data not shown). The ratio of bound radioactivity between the respective BRSV G ORFs and the corresponding BHV-1 gD ORFs was 1.7 with RNA from BHV-1/eG_{ori}-infected cells and 2.1 with RNA from BHV-1/eG_{syn}-infected cells, demonstrating that the G_{ori} ORF was transcribed in BHV-1/eG_{ori}-infected cells. These results are in accordance with the conclusion that BHV-1-expressed BRSV G_{ori} RNAs are unstable in the nuclei of infected cells.

Identification and characterization of BHV-1-expressed BRSV G glycoprotein. To test for the expression of the BRSV G glycoprotein in BHV-1/eG_{syn}-infected cells, BRSV G glycoprotein-specific MAb 20 and a polyclonal antiserum (anti-VacG_{syn}), raised in rabbits after infection with G_{syn}-expressing recombinant vaccinia virus, were used to stain wild-type

BHV-1 strain Schönböken (BHV-1/Schö)-, BHV-1/eG_{syn}-, or BHV-1/eG_{ori}-induced plaques by indirect immunofluorescence. The cultures were fixed 2 days after infection and incubated with the BHV-1 gD-specific MAb 21/3/3, MAb 20, or the anti-VacG_{syn} serum. Antibodies bound on the cell surface were visualized by using DTAF-conjugated anti-species immunoglobulin G and fluorescence microscopy. As shown in Fig. 5, MAb 20 and the anti-VacG_{syn} serum bound only to cells in plaques generated by BHV-1/eG_{syn} whereas MAb 21/3/3 reacted with gD on the surface of cells infected with BHV-1/Schö, BHV-1/eG_{syn}, and BHV-1/eG_{ori}. The conclusion that the BRSV G_{syn} ORF-encoded protein is expressed on the surface of BHV-1/eG_{syn}-infected cells was also demonstrated by flow cytometry with living cells and the same antibodies as above (data not shown).

To further characterize the BRSV G_{syn} ORF-encoded gene product, proteins from infected cells (Fig. 6, lanes 1 to 4), purified virions (lanes 5 to 7), and the cell culture medium from BHV-1/eG_{syn}-infected cells (lane 8) were analyzed by immunoblotting because MAb 20 and the anti-VacG_{syn} serum did not work for immunoprecipitation. MAb 20 did not specifically bind to proteins from cells infected with BHV-1/Schö (Fig. 6, lane 1) and BHV-1/eG_{ori} (lane 2) or to proteins from purified BHV-1/Schö and BHV-1/eG_{ori} virions (lanes 5 and 6) but reacted strongly with proteins with apparent molecular masses of 38 and 43 kDa from BHV-1/eG_{syn}-infected cells (lane 3). In addition, several weaker-staining bands ranging in size from 70 to about 100 kDa were detected. To elucidate

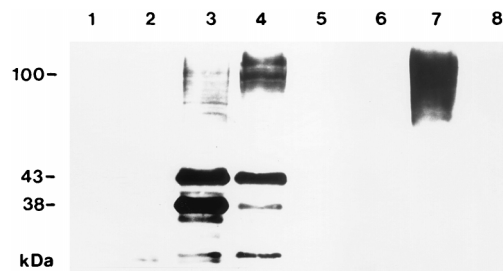


FIG. 6. Identification of the BHV-1/eG_{syn}-expressed BRSV G glycoprotein. MDBK cells were infected with BHV-1/Schö (lanes 1 and 5), BHV-1/eG_{ori} (lanes 2 and 6), and BHV-1/eG_{syn} (lanes 3, 4, 7, and 8). Proteins from infected cells, harvested at 10 h p.i. (lanes 1 to 4), from purified virions (lanes 5 to 7), and from cell culture medium (lane 8), were analyzed by immunoblotting with BRSV G glycoprotein-specific MAb 20. Proteins shown in lane 4 were from cells incubated with cycloheximide (100 µg/ml) for 2 h before lysis. Apparent molecular masses are indicated in kilodaltons.

possible precursor-product relationships among the MAb 20-reactive polypeptides, BHV-1/eG_{syn}-infected cells were incubated for 2 h with cycloheximide (final concentration, 100 µg per ml) before lysis to inhibit *de novo* protein synthesis. This treatment resulted in a considerable decrease in the signal at 38 kDa and a slight reduction in the band intensity of the signal at 43 kDa, whereas the bands corresponding to larger proteins increased in size and abundance (Fig. 6, lane 4). Prolongation of the cycloheximide incubation time did not significantly alter this pattern but resulted in a decrease in the intensities of all bands (data not shown). Among proteins from purified virus particles, MAb 20 bound only to polypeptides from BHV-1/eG_{syn} virions which migrated as a diffuse band with an apparent molecular mass between 70 and 120 kDa (lane 7). No MAb 20-reactive proteins were detected in the medium of BHV-1/eG_{syn}-infected cells (lane 8). Identical results were obtained with the anti-VacG_{syn} serum and BRSV G glycoprotein-specific MAb 57 (9). The latter immunoprecipitated the large molecules but not the 38- and 43-kDa proteins from cells infected with BHV-1/eG_{syn} in the presence of [³⁵S]methionine and [³⁵S]cysteine. From these results, we conclude that the 38- and 43-kDa proteins represent BRSV G glycoprotein precursor molecules which are subsequently modified to the large form, which is also associated with BHV-1/eG_{syn} virions.

The carbohydrate composition of virus particle-associated BRSV G glycoprotein was analyzed by incubation of purified BHV-1/eG_{syn} virions with glycosidase F, neuraminidase, and neuraminidase together with *O*-glycosidase followed by immunoblotting after SDS-PAGE with MAb 20 (Fig. 7a) or a polyclonal rabbit serum raised against purified BHV-1 gD (Fig. 7b) to monitor the reactions. According to the enzymes used, the electrophoretic mobilities of virion-associated gD increased as expected (41), and no differences were detected in comparison to the reaction products of immunoprecipitated virion gD (Fig. 7b and c), indicating the presence of sufficient enzymatic activity for complete deglycosylation within the respective probes. The BRSV G glycoprotein molecules appeared unaffected after incubation with neuraminidase (Fig. 7a, lane 2) and exhibited an increased electrophoretic mobility following digestion with neuraminidase and *O*-glycosidase (lane 3) or *N*-glycosidase F (lane 4), which demonstrates the presence of *O*- and *N*-linked carbohydrates in the virion-associated BRSV G glycoprotein. However, digestion products obtained after incubation of the virion proteins with neuraminidase and *O*-glycosidase migrated slower and were more diffuse, as expected for the only *N*-glycosylated form. Whether this discrep-

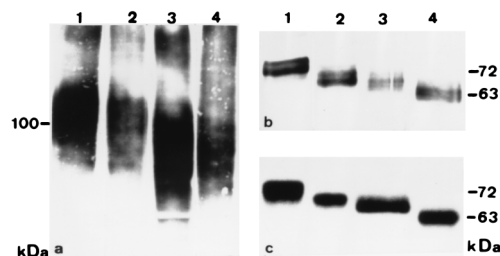


FIG. 7. Evidence that BHV-1-expressed G glycoprotein contains *N*- and *O*-linked sugars. (a and b) Purified BHV-1/eG_{syn} virions were resuspended in PBS plus 0.5% NP-40 and digested overnight at 37°C with neuraminidase (lanes 2), neuraminidase and *O*-glycosidase (lanes 3), *N*-glycosidase F (lanes 4), and without enzyme (lanes 1). Digestion products were analyzed by immunoblotting with G glycoprotein-specific MAb 20 (a) or a polyclonal, BHV-1 gD-specific rabbit antiserum (b). (c) Purified, [³⁵S]methionine-labeled BHV-1/Schö virions were lysed and incubated with gD-specific MAb 21/3/3. Immunoprecipitated proteins were incubated as described above, separated on SDS-10% polyacrylamide gels, and visualized by fluorography. The apparent molecular masses, indicated in kilodaltons on the left, were calculated from the migration of prestained (a and b) or [¹⁴C]-methylated protein molecular mass standards (c) run on the respective gel.

ancy is due to additional modifications, the presence of *O*-glycosidase-resistant *O*-linked carbohydrates (22), or incomplete removal of *O*-linked glycans due to specifics of the BRSV G glycoprotein has to be analyzed.

Virus neutralization assays. The immunoblot analysis in Fig. 6 indicated that BHV-1/eG_{syn} virus particles contain the BRSV G glycoprotein and therefore should be susceptible to inactivation by BRSV G glycoprotein-specific antibodies. BHV-1/eG_{ori} and BHV-1/eG_{syn} virions were tested for complement-dependent and -independent neutralization by MAb 57, the anti-VacG_{syn} serum, and the anti-BRSV serum 2106 (9). With complement, MAb 57, which had no effect on the infectivity of BHV-1/eG_{ori} (data not shown), reduced the number of BHV-1/eG_{syn}-induced plaques to 52% at a final dilution of 1:25. The anti-VacG_{syn} serum specifically neutralized BHV-1/eG_{syn} in the presence of complement to 50% neutralization at a dilution of about 1:1,000 (Fig. 8). No activity was detectable without complement (data not shown). The BHV-1/eG_{syn}-neutralizing activity of anti-BRSV serum 2106 was weak in the absence of complement and more pronounced after the addition of complement. Both polyclonal sera had no significant effect on the infectivity of BHV-1/eG_{ori} (Fig. 8) and BHV-1/Schö (data not shown).

DISCUSSION

Analysis of the expression from the authentic BRSV G glycoprotein ORF after infection of cells with BHV-1/eG_{ori} indicated that the transcripts are unstable in the nucleus. We assumed that this was due to the nucleotide sequence of the RNA, which might contain signals that do not interfere with the stability of the RNA in the cytoplasm of cells infected with BRSV or recombinant vaccinia virus expressing the BRSV G glycoprotein (24) but result in degradation of the transcripts in the nucleus. Therefore, the complete BRSV G glycoprotein ORF was reconstructed by using synthetic oligonucleotides. The codon usage of the modified ORF was adjusted to that of BHV-1 gD, resulting in an increase of the G+C content from 42 to 62%, and the introduction of putative splice donor signals (30) was avoided. The synthetic ORF was inserted into the genome of BHV-1 under the control of the MCMV e1 promoter (2, 21), and analysis of the gene products in BHV-1/eG_{syn}-infected cells demonstrated transcription of mRNA,

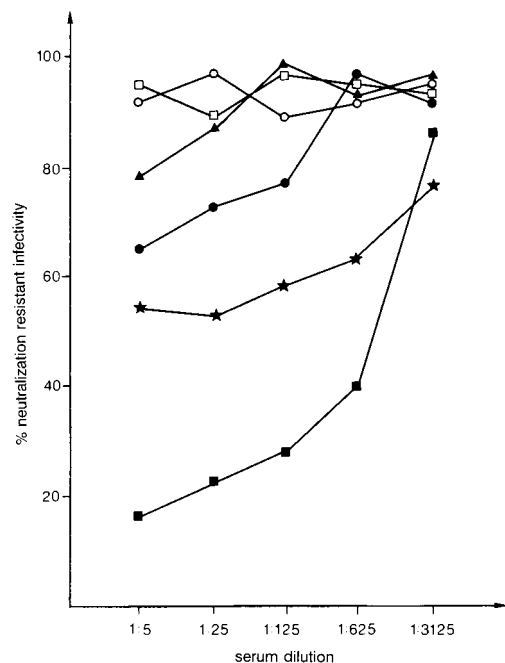


FIG. 8. BHV-1/eG_{syn} virions are susceptible to neutralization by antibodies against the BRSV G glycoprotein. Approximately 200 PFU of BHV-1/eG_{ori} was incubated with 20% fetal calf serum plus complement or serial dilutions of anti-VacG_{syn} with complement (open squares) or the anti-BRSV hyperimmune serum with complement (open circles), and about 160 PFU of BHV-1/eG_{syn} was incubated with 20% FCS and complement or with serial dilutions of anti-VacG_{syn} and complement (solid squares), MAb 57 with complement (stars), anti-BRSV serum 2106 without complement (solid triangles, no complement added to the FCS control), or anti-BRSV serum 2106 with complement (solid circles). After 60 min at 37°C, the virions were plated on MDBK cells and the cultures were overlaid with semisolid medium. Plaques were counted 3 days later. The plaque count of each FCS control was defined as 100% neutralization-resistant infectivity. Serum dilutions are indicated.

which was translated into proteins recognized by BRSV G glycoprotein-specific MAb 20 and the anti-VacG_{syn} serum. Although we did not attempt to delineate sequence motifs leading to instability of the G_{ori} transcripts in the nucleus, our results strongly suggest their existence, which might also explain the failure to express the authentic ORF encoding BRSV fusion protein F by BHV-1 (13). Introduction of a series of single restriction enzyme cleavage sites into the G_{syn} ORF will be helpful in identifying sequence elements that prevent the generation of mRNA from the genomic BRSV G glycoprotein ORF, which may be beneficial for the development of systems for the expression of RNA virus genes in the nuclei of mammalian cells.

The BRSV G glycoprotein is a type II membrane glycoprotein with a 37-amino-acid N-terminal cytoplasmic domain followed by 29 hydrophobic amino acids representing the transmembrane region. The remaining 191 amino acids are mainly hydrophilic and constitute the extracellular domain (24). Among BHV-1/eG_{syn}-infected cell proteins, MAb 20, MAb 57, and the anti-VacG_{syn} serum detected proteins with apparent molecular masses of 38, 43, and 70 to 120 kDa. Inhibition of de novo protein synthesis by cycloheximide resulted in a decrease of the signals generated by the 38- and 43-kDa proteins and a concomitant increase in intensity and size of the large-protein band. Although the exact precursor-product relationship awaits clarification, we assume that the 38- and 43-kDa proteins represent precursor molecules of the mature BRSV G glycoprotein. This assumption is in accordance with results

reported by Lerch et al. (24), who demonstrated that in cells infected with a BRSV G glycoprotein-expressing recombinant vaccinia virus, two proteins which migrated with an apparent molecular mass of 43 kDa and as a broad band between 68 and 97 kDa specifically reacted with an anti-BRSV serum in immunoblots. They further provided evidence that the 43-kDa form represents a precursor containing only N-linked carbohydrates whereas the 68- to 97-kDa molecules constitute the mature N- and O-glycosylated BRSV G glycoprotein.

Expression of the BRSV G glycoprotein in BHV-1/eG_{syn}-infected cells was analyzed only by immunoblotting because the BRSV G glycoprotein-specific MAb 20 and the anti-VacG_{syn} serum did not work for immunoprecipitation and MAb 57 precipitated only the mature glycoprotein after synthesis in the presence of [³⁵S]methionine and [³⁵S]cysteine (not shown). The observation that antibodies against the BRSV G glycoprotein bind in immunoblots but fail to immunoprecipitate the antigen is, although surprising, not without precedent and has also been reported by Lerch et al. (24). The reason for this apparent paradox, however, remains unclear.

Immunofluorescence and flow cytometry (data not shown) with MAb 20 and the anti-VacG_{syn} serum demonstrated that BHV-1/eG_{syn}-infected cells expressed the BRSV G glycoprotein on the cell surface, and we conclude that it is tightly anchored in the cell membrane since no secreted molecules were found in the medium from BHV-1/eG_{syn}-infected cultures. Thus, our results indicate Golgi processing and post-Golgi transport of the BHV-1/eG_{syn}-encoded BRSV G glycoprotein to the cell surface, comparable to the situation in BRSV- or recombinant vaccinia virus-infected cells (24).

Since alphaherpesviruses probably do not encode type II glycoproteins, we investigated whether the BRSV G glycoprotein that presumably lacks any herpesvirus-specific targeting signals is incorporated into BHV-1 particles. Western blot analysis with MAb 20 revealed incorporation of the BRSV G glycoprotein into purified virus particles, and this was confirmed by the susceptibility of BHV-1/eG_{syn} virions to neutralization by the anti-VacG_{syn} serum, the anti-BRSV immune serum, and MAb 57 in the presence of complement. That these antibodies did not neutralize in the absence of complement was not too surprising, since it is unlikely that the BRSV G glycoprotein exhibits a biological function for the infectivity of BHV-1. In addition, flow cytometry demonstrated that MAb 20 and the anti-VacG_{syn} serum bound to living cells infected with purified BHV-1/eG_{syn} virions in the presence of cycloheximide to prevent de novo protein synthesis (13). This further supports the conclusion that the orientation of the BRSV G glycoprotein within the viral envelope is correct.

To our knowledge, this is the first description of a class II membrane glycoprotein becoming incorporated into alphaherpesvirus virions. The presence of the BRSV G glycoprotein in the given background of BHV-1/eG_{syn} exerted only a slight influence on the entry of virions into target cells in comparison to the situation for wild-type BHV-1 (data not shown). Although no repaired viruses were included in this study, we believe that it is reasonable to assume that BRSV G glycoprotein does not significantly interfere with the envelope structures required for the initial steps of the BHV-1 infection.

This study has practical implications for herpesvirus-based vector vaccine development: (i) it indicates that modification of RNA virus genes may be required for efficient expression, and (ii) it raises the possibility of targeting foreign antigens as class II membrane (glyco)proteins into the envelope of herpesvirus virions, which might be important when booster vaccinations with live recombinant viruses are inefficient due to

existing immunity to the vector, a situation observed with recombinant vaccinia viruses (13, 26).

ACKNOWLEDGMENTS

We thank Julie Furze for providing MAb 57, Helmut Stephan and Elke Zorn for photography, J. Naval for corrections, and Bernd Köllner for assistance with the flow cytometry.

Financial support for synthesis and expression of the G_{syn} ORF by Intervet International BV is greatly appreciated. G.K. was supported by grant BIOT-CT93-0489 from the Commission of the European Communities.

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